



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup>:</b> C12Q 1/68, B01L 3/00, 3/14	<b>A1</b>	<b>(11) International Publication Number:</b> WO 96/26291 <b>(43) International Publication Date:</b> 29 August 1996 (29.08.96)
<b>(21) International Application Number:</b> PCT/GB96/00420 <b>(22) International Filing Date:</b> 23 February 1996 (23.02.96)  <b>(30) Priority Data:</b> 9503808.9      24 February 1995 (24.02.95)      GB  <b>(71) Applicant (for all designated States except US):</b> THE UNIVERSITY OF NOTTINGHAM [GB/GB]; University Park, Nottingham NG7 2RD (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> BARDSLEY, Ronald, George [GB/GB]; 15 Banbury Drive, Shepshed, Leicestershire LE12 9PL (GB). LOCKLEY, Andrew, Keith [GB/GB]; 16 Blanchcroft, Melbourne, Derbyshire DE73 1BA (GB). FRANKLIN, Stephen, Joseph [GB/GB]; 4 Radnor Drive, Shepshed, Leicestershire LE12 9SA (GB).  <b>(74) Agent:</b> PRIVETT, Kathryn, Louise; Stevens, Hewlett & Perkins, 1 Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB).		<b>(81) Designated States:</b> JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> MUTATION DETECTION USING SOLID PHASE PCR		
<b>(57) Abstract</b>  A method of detecting mutations, which comprises amplifying target DNA using two related primers immobilised at spaced locations on a support and a third primer capable of hybridising to extension products of the first two primers, and detecting amplified target sequence on the support by means of a label incorporated in the extension products.		

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## Mutation Detection Using Solid Phase PCR

This invention relates to detection of the presence of alternative DNA sequences at a specific locus on a DNA strand. More particularly, the invention relates to determining the presence or  
5 absence of mutant or corresponding wild type DNA sequences. The invention relates also to apparatus for performing solid-phase nucleic acid amplification.

DNA analysis is becoming important in a broad spectrum of areas. Forensic applications for DNA  
10 fingerprinting have met with success. Equally valuable are improved methods of screening for genetic markers related to desirable traits in livestock, disorders such as muscular dystrophy and cystic fibrosis, and  
15 diagnosis of bacterial and viral infections. Another area of increasing importance is DNA analysis in meat and fish product speciation, since food adulteration persists in some parts of the industry.

Most common methods for presenting genetic information in these areas involve DNA fragment  
20 analysis including DNA fingerprinting. Despite being widely used, such techniques remain fairly sophisticated, requiring advanced laboratories and skilled personnel. Much of this stems from the need to extract DNA or its fragments, separate these on fragile  
25 gel systems, and detect components of interest present in minute amounts using radioisotopes.

In contrast, many diagnostic techniques based on immunochemistry, the use of antibodies to detect specific proteins, have progressed to a stage where  
30 small laboratories, doctors or food analysts readily operate these methods, and pregnancy testing kits can even be purchased by the general public. There is an increasing demand to make DNA analysis more accessible to hospitals, forensic or food laboratories to enable  
35 them to screen for genetic factors in a simple one- or

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two-step operation with minimal equipment.

A point mutation known as the "halothane" mutation in the porcine ryanodine receptor gene is associated with the disease porcine malignant hyperthermia (MH). In animals which are homozygous for the halothane gene, inhalational anaesthetics such as halothane trigger skeletal muscle contraction with attendant hypermetabolism and an elevation in body temperature. The disease can also be triggered by stress in homozygous animals. The result is pale, soft, exudative (PSE) pork, and major economic losses in the industry. However, controlled existence of the halothane gene may be of benefit to the pig industry because heterozygous animals have significantly superior carcass traits compared with homozygous normal pigs. The currently used diagnostic test for MH involves PCR to amplify the region of DNA either side of the potential mutation site, and the presence or absence of the mutation can subsequently be demonstrated by a restriction endonuclease digestion assay and electrophoresis, because the halothane mutation changes a restriction enzyme recognition sequence. The test requires highly technical sample manipulation by skilled personnel. Additionally, it is not completely reliable, as occasionally the restriction enzymes may not cut suitable fragments of DNA, and internal controls need to be present in every reaction. Even if the DNA is cut, if not all of the PCR-amplified fragments are cut completely, those remaining fragments may produce a faint band when visualised on a gel, resulting in the test being suggestive of a heterozygous genotype in a homozygous individual. Simpler and more reliable testing methods are therefore required.

WO 90/11372 describes a method for determining the existence or not of a particular

nucleotide at a particular position on a strand of DNA. Primers specific for that nucleotide and having unique tag sequences are employed in an extension reaction. Any resulting extended primers are then immobilised on  
5 a support via the tag sequence, for detection. A series of different primers can be used to investigate a number of different alleles at the same time, as long as each different primer is provided with its own unique tag sequence.

10 WO 90/11369 describes a detection method for diagnosis of genetic medical conditions, which involves amplifying a part of the DNA strand of interest using a first pair of liquid phase primers specific to the target DNA, and then further amplifying using a nested  
15 pair of primers, one of which is immobilised or immobilisable.

Various non gel-based tests are being developed for looking at genetic markers. Much current research in this area utilises "strip technology", in  
20 which solid supports with spatially separated probes recognise and trap specific DNA products without the need for gel electrophoresis. In all tests of this type so far developed or known to be in development, the solid support entraps DNA products resulting from  
25 an earlier, separate step. One example of such a technique is the Line Probe Assay of Dr Rudi Rossau, Innogenetics, Belgium, in which specific oligonucleotides are immobilised as parallel lines on membrane based strips. Biotin-labelled amplified  
30 targets are hybridised with the oligonucleotides, and binding is visualised using streptavidin conjugated with alkaline phosphatase and a BCIP/NBT chromogen. Strips are marketed for a variety of clinical applications and have between 9 and 32 markers. A test  
35 time from PCR-prepared DNA to result could take 5 hours for one multiparameter test. The PCR reaction and

product analysis by capture probe remain separate operations, which makes the test less easy to use than a one step reaction.

The present invention provides in one aspect,  
5 a method for detecting the presence or absence of two alternative target sequences at a specific locus on strands of DNA in a sample which method comprises  
amplifying the target sequence or sequences present in the sample by means of two related primers  
10 used under conditions such that each of said primers is capable of hybridising to only one of the two alternative target sequences and of forming an extension product, and a third primer which is an antisense primer capable of hybridising to the  
15 extension product and of forming a complementary extension product,

wherein the two related primers are present immobilised at spaced locations on a support and a detectable label is incorporated in the extension  
20 product such that amplified target sequence can be detected on the support.

Preferably but not necessarily, the alternative target sequences are a wild type allele and a mutant allele of the same gene, in which case the  
25 mutation is preferably a point mutation, thus the target sequences will differ at a single nucleotide position. Alternatively, the target sequences could be from the same gene in different animals and differ from each other at several or preferably one nucleotide  
30 position(s).

In one embodiment of the method, a difference between the two immobilised primers at their 3' ends gives the primers specificity for a particular target sequence. The principle of the amplification refractory  
35 mutation system (ARMS) may be applied (Newton, C.R. et al (1989) Nucl. Acids Res., 17, 2503-2516). ARMS works

on the principle that oligonucleotides which are complementary to a given DNA sequence except for a mis-match at their 3' termini fail to function as primers in PCR, and it may be a general technique for the analysis of any point mutation or small deletion.

The principle of the mutagenically separated polymerase chain reaction (MS-PCR) may also be applied in the method according to the invention (Rust, S. et al (1993) Nucl. Acids Res., 21, 3623-3629). In an MS-PCR adaptation, the two immobilised primers differ as in ARMS, but are also different to one another in respect of one or more further nucleotides within the terminal few, eg. four, 3' nucleotides. Thus, each primer is mis-matched with its target sequence with respect to one or more nucleotides and those mis-matches differ from mis-matches of the other primer and its target sequence.

Advantageously, there may be present in the liquid phase non-immobilised primers corresponding partially or wholly to one or both of the immobilised primers. Such liquid phase primers can act to enhance signal strength and improve discrimination of the primers between their specific target and the other target.

In a further aspect, the invention provides a method of testing for the presence of the halothane mutation in a sample containing DNA from an animal, which method comprises,

performing an amplification reaction on the DNA using the PCR primers:

(i) MS-P1: 5'-CCT GTG TGT GTG CAA TGG TGT GGC CGT CC -3'

(ii) MS-P2: 5'-GTG CTG GAT GTC CTG TGT TCA ATG TGT GTG TGC AAT GGT GTG GCC GGG T -3'

and an antisense primer which hybridises to a DNA strand downstream of (i) and (ii), separating the

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amplification products and observing the separated products as an indication of the presence of one or more copies of the halothane mutation.

In a still further aspect, the invention provides apparatus for nucleic acid manipulations comprising a microtitre vessel, such as a microcentrifuge tube, having a closed lower end, to contain a volume of liquid which is small relative to the total internal volume of the vessel, and an open top which is closeable by a lid, the lid carrying a plug which extends into the tube and abuts the side wall thereof to define a liquid-containing region adjacent the lower end of the tube, wherein there is provided an insert mounted on the plug so as to be positioned in the said liquid-containing region of the tube, the insert being adapted to carry one or more immobilised nucleic acid deposits.

Preferably, the plug reduces the internal volume of the vessel by at least half. The reduction in volume is designed to prevent problems associated with the heating steps carried out during amplification. The insert is essentially a support for immobilised oligonucleotides.

The support carrying the primers may be comprised of any suitable material to which nucleic acid primers may be immobilised. An inert support material to which nucleic acids can be covalently attached by ultraviolet cross-linking, such as Hybond N (Amersham International, UK) is suitable. A poly T sequence at the 5' end of the primers can be linked irreversibly to such nylon-based nitrocellulose membranes. Primers can alternatively be immobilised in other ways known in the art.

The immobilised primers will need to be designed according to the particular target DNAs being investigated. The target DNA sequences need to be

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known DNA sequences with identified differences between them. The invention is not however limited to only two different immobilised primers. There may be a range of primers, for testing for a range of different mutations or for a range of different species. In the case where testing is for more than one mutation, liquid-phase non-selective primers may be needed to provide an antisense primer for each mutation investigated.

The antisense primer in the solid-phase PCR method according to the invention is not discriminatory between the different target sequences. It hybridises to the targets downstream of the immobilised primers and to the opposite strand. The length of the amplification products will depend on the relative positions on the targets to which the immobilised primers and the antisense primer hybridise, and is not critical to the invention but may need to be taken into consideration, depending on the method of detection which is used. Generally, the extension products need to be long enough to incorporate sufficient label to be detectable. For example, the extension products may be at least 100 bases in length. A preferred range for the length of the extension products is 100-1000 bases.

Suitable lengths for the primers will be known to those skilled in the art. Immobilised primers linked to the support via a polyT sequence may require a polyT tail of for example 80 - 100 bases for optimal immobilisation by cross-linking. Non-immobilised counterparts to the immobilised primers are normally in the range of 20 to 30 base pairs, but are not limited to those lengths.

With non-immobilised counterparts to the immobilised primers present in the reaction mixture very much better results are possible. The non-immobilised counterparts are thought to act in cooperation with the antisense primer by a "kick-start"

type mechanism. More template target DNA becomes available to the immobilised primers as a result of PCR amplification by the liquid-phase primers. Precise adjustment of concentrations of the respective primers will be necessary to optimise results and is within the capability of a person skilled in the art. Preferably, using non-immobilised counterparts of the immobilised primers should not complicate the method for the user because amplification will still be performed with all reagents present in a single step. The "kick-start" effect may alternatively be achieved by using a liquid-phase primer which hybridises to the target DNA on the 5' side (upstream) of the immobilised primers. This approach constitutes a half-nested PCR.

The detectable label incorporated into the extension product may be any suitable label. It can be either directly detectable eg. fluorescent, or detectable by means of further reagents. One labelling system which works particularly well is the DIG visualisation system (Boehringer Mannheim), in which the steroid hapten digoxigenin linked to deoxyuridine triphosphate, which is a suitable substrate for polymerase enzymes eg. Taq DNA polymerase, is incorporated into PCR amplification products. The labelling system is preferably one which allows development of a visible signal by simple steps. The DIG system achieves this; a support with amplified target attached can be simply placed in the appropriate solutions for the immunochemical detection system according to the prescribed manufacturers' procedure. By this system, it has been possible to obtain clear signals by the solid phase method in under two hours. Other labelling systems may be equally good.

Samples for use in the assay method according to the invention may be DNA samples extracted for

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example from blood leukocytes. Various DNA extraction methods are well-known in the art and simple kits and techniques are available. Extraction methods can be optimised for use by unskilled personnel. The sample may alternatively be of whole blood, according to the method of Burckhardt, J. (1994) PCR Methods and Applications, 3, 239-243.

The target DNA in the method according to the invention is preferably but not necessarily double-stranded genomic DNA. However other sources of nucleic acid such as cDNA could be used.

The target amplification may conveniently be performed in a microcentrifuge tube such as an eppendorf tube, or any other vessel designed for holding small quantities of liquid. Alternatively, a flat-bed format may be used, such as that described in co-pending Application No. GB 9411172.

Reference is directed to the accompanying drawings in which:

Figure 1 is a schematic representation of the liquid-phase MS-PCR system for the halothane gene;

Figure 2 shows results of MS-PCR performed on dipsticks, as described in Example 1.

Figure 3 is a diagrammatic representation of a vessel with plug and insert, according to the invention;

Figure 4 shows the results of the comparison of a restriction-based diagnostic test with MS-PCR for the detection of the halothane mutation, as described in Example 2.

In figure 3, a vessel 30 such as an Eppendorf tube is provided for receiving a small volume of liquid 31. The internal volume of the tube is considerably

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greater than the volume of the liquid (100 $\mu$ l as shown) in order to facilitate manipulation. The tube has a conical closed lower end and an open top which is shown closed by a lid 32. A plug 33 greatly reducing the internal volume of the tube extends from the lid into the tube. The lower extremity of the plug is set tightly against the internal conical part of the tube at 34. This vessel is described in more detail in WO 90/00442.

Mounted at the bottom end of the plug by means of an arm 35 is an insert 36 which is a pennant-shaped piece of nitrocellulose membrane to act as a support for one or more immobilised primers for performing solid-phase nucleic acid amplification reactions. In use the insert 36 is immersed in or in contact with the liquid 31.

The invention will now be further described by means of the following examples.

## EXAMPLES

### Example 1

Application of mutagenically separated PCR to detect the porcine halothane mutation using dipstick technology

### Primers

The halothane mutation occurs at position 1843 in the ryanodine receptor cDNA sequence (Fujii et al (1991) Science, 253, 448-451). The last three bases up to and including no. 1843 are 5'- TGC -3' in the normal, wild type sequence and 5'- TGT -3' in the mutant sequence.

The following two primers were synthesised

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for immobilisation, each having a polythymidine anchor of 81 T's at the 5' end:

5 (i) KITMS-P1, a wild type allele-specific primer:

5'- CCT GTG TGT GTG CAA TGG TGT GGC CGT CC -3'.

The penultimate base at the 3' end of KITMS-P1 is a mismatch for the normal ryanodine receptor sequence (a G to C alteration).

10 (ii) KITMS-P2, a mutant allele-specific primer consisting of a poly T anchor of 81 thymidines at the 5' end and the primer sequence:

5'- AAT GTG TGT GTG CAA TGG TGT GGC CGG GT -3'.

15 The third base from the 3' end introduces a mismatch for the mutant ryanodine receptor sequence (a T to G alteration). Also, the two 5' adenines are mismatches (both are C to A alterations).

20 Thus each of the primers KITMS-P1 and P2 contains a deliberate mismatch at or near the 3' end, but each is in a different position, so that the PCR products of these primers will differ in 3 out of their last 4 bases. This reduces cross-reactions in PCR cycling as the products are "mutated" away from the original genomic template DNA sequence. The two  
25 additional mismatches at bases 82 and 83 of KITMS-P2 inhibit the forming of heteroduplex molecules.

30 A non-selective (for the wild type or mutant sequence) complementary strand primer was chosen at a distance from the MS-PCR primers such that the products synthesised had a length of 114 base pairs. The non-selective primer was as follows:

(iii) MS-P3

5'- CTG GTG ACA TAG TTG ATG AGG TTT GTC TGC -3'.

35 Additional primers MS-P1 and MS-P2, corresponding to KITMS-P1 and KITMS-P2 but without the

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poly T anchor were synthesised, for use as liquid phase primers.

## 5     Dipsticks

Dipsticks comprised Hybond N (Amersham) inserts cut so as to fit into a solution PCR reaction, with the primer KITMS-P2 immobilised as a discrete spot on to the bottom of the nylon, and KITMS-P1 immobilised as a discrete spot above this. A small volume, 0.2  $\mu$ l of a 20pmol  $\mu$ l<sup>-1</sup> solution of each primer was spotted onto the Hybond using a Gilson Pipetman P2, and the oligonucleotides were covalently attached to the membrane by UV-crosslinking (Stratagene UV Stratalinker 1800, "autocrosslink" setting, giving a preset exposure of 1200 microjoules).

## Amplification

Genomic porcine DNA was obtained from blood samples supplied by JSR Healthbred, by extraction of the DNA from leucocytes by published procedures (O'Brien, P.J. et al (1993) JAVMA, 203, 842).

Reactions contained the following: 10mM Tris-HCl (pH8.8 at 20°C); 1.5mM MgCl<sub>2</sub>; 50mM KCl; 0.1% Triton X-100; 6.19  $\mu$ M each of dATP, dCTP, dGTP; 5.79  $\mu$ M dTTP; 0.21  $\mu$ M Dig-dUTP; 0.1  $\mu$ M MS-P3; 0.08  $\mu$ M MS-P2; 0.8  $\mu$ M MS-P1; 125ng porcine DNA; 1.5 Units Taq DNA polymerase (Boehringer Mannheim) added in a Hot Start; sterile distilled water to 100  $\mu$ l. A dipstick was added to each reaction, prior to the addition of an overlay of 50  $\mu$ l sterile mineral oil to prevent evaporation during the thermal cycling. The PCR reaction conditions did not employ the standard polymerase buffer routinely supplied by Boehringer Mannheim, which contained 0.1mg ml<sup>-1</sup> gelatine; the substitution of 0.1% Triton X-100 for gelatine was found to permit the development of

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colour associated with discrete spots of immobilised product.

Thermal cycling conditions were: 1x[95°C 5 min; 69°C 1 min (during which time the Taq was added); 72°C 1 min] followed by 35x [95°C 30 sec; 69°C 45 sec; 72°C 45 sec]. Immediately the cycling was finished, the dipsticks were removed from the tubes containing the reactions and subjected to immunochemical detection of the DIG hapten according to a procedure described by Boehringer Mannheim. Routinely, clear signals were apparent on the dipsticks within 5-10 minutes.

Dipsticks used against homozygous mutant DNA had a discrete purple spot where the mutant-specific primer had been attached and were otherwise blank (Figure 2A). Dipsticks used against homozygous wild type DNA had a discrete purple spot where the wild type-specific primer had been attached and were otherwise blank (Figure 2C). Use of DNA from heterozygotes resulted in both spots being coloured (Figure 2B).

## Example 2

### Comparison of restriction-based diagnostic test with liquid-phase MS-PCR for the detection of the porcine halothane gene

#### Primers

Three primers were used for the MS-PCR method:

- (i) MS-P1.
- (ii) The following long mutant allele-specific primer similar to MS-P2:  
5'- GTG CTG GAT GTC CTG TGT TCA ATG TGT GTG TGC AAT GGT GTG GCC GGG T -3'.
- (iii) MS-P3 as the non-selective complementary primer.

### Methods

Porcine DNA was obtained from isolated leucocytes, as in Example 1. For the restriction-based test, PCR primers, reaction conditions and cycling parameters were as described previously (Houde, A. and Pommier, S.A. (1993) Meat Science, 33, 349-358) but with a reaction volume of 50 $\mu$ l rather than 25 $\mu$ l.

For the MS-PCR method, 50 $\mu$ l reactions were set up containing 125 $\mu$ g DNA, 20 $\mu$ M dATP, dCTP, dTTP and dGTP (Boehringer Mannheim), 0.1 $\mu$ M of the complementary strand primer, 0.08 $\mu$ M of the mutant allele-specific primer, 8 $\mu$ M of the wild-type allele-specific primer, 10mM Tris-HCl (pH8.3 at 25°C), 1.5mM MgCl<sub>2</sub>, 50mM KCl, 0.1mg/ml gelatine and 1.25 Units of Taq DNA polymerase (Boehringer Mannheim). These were overlaid with 50 $\mu$ l liquid paraffin. The amplification was performed using a Hybaid Omnigene thermal cycler, with a "Hot Start" [95°C 5 mins, 69°C 1 min, 72°C 1 min] followed by 40 x [95°C 30s, 69°C 45s, 72°C 45s], and 1 x [72°C 3 min]. 20 $\mu$ l of the product was analysed on a 4% MetaPhor agarose (FMC Bioproducts) minigel.

Figure 4 shows the products separated in the gel, in which the samples and treatment for each lane were as follows: lane 1: size standards (HaeIII digest of  $\phi$ X174), lane 2: homozygous wild-type DNA sample (49bp and 32bp fragments resulting from 81bp HhaI cut PCR product), lane 3: homozygous mutant DNA sample (81bp product uncut by HhaI), lane 4: heterozygote DNA sample (both uncut (81bp) and cut (49bp and 32bp) fragments), lane 5: homozygous wild-type DNA sample (114bp product derived from (short) wild-type allele specific primer), lane 6: homozygous mutant DNA sample (134bp product derived from (long) mutant specific primer), lane 7: heterozygous DNA sample (114bp and 134bp products, derived from both allele-specific primers), lane 8: size standards (HaeIII

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digest of  $\phi$ X174). Lanes 2, 3 and 4 are thus the products of the restriction-based test and lanes 5, 6 and 7 are MS-PCR products.

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## CLAIMS

1. A method for detecting the presence or absence of two alternative target sequences at a specific locus on strands of DNA in a sample which method comprises  
5 amplifying the target sequence or sequences present in the sample by means of two related primers used under conditions such that each of said primers is capable of hybridising to only one of the two  
10 alternative target sequences and of forming an extension product, and a third primer which is an antisense primer capable of hybridising to the extension product and of forming a complementary  
15 extension product,  
wherein the two related primers are present immobilised at spaced locations on a support and a detectable label is incorporated in the extension product such that amplified target sequence can be  
20 detected on the support.
2. A method as claimed in claim 1, wherein one target is from a wild type allele and the other is from a mutant allele, of the same gene.
3. A method as claimed in claim 1 or claim 2,  
25 wherein the alternative target sequences differ from one another at a single nucleotide position.
4. A method as claimed in any one of claims 1 to 3, wherein the two immobilised primers have different nucleotide bases at their 3'-end, for base-pairing with  
30 a different nucleotide base on each target sequence.
5. A method as claimed in claim 4, wherein the two immobilised primers differ at one or more further nucleotides within their terminal four 3'-nucleotides and those nucleotides are mis-matches with respect to  
35 each target sequence.
6. A method as claimed in any one of claims 1 to

5, wherein further primers corresponding to the two immobilised primers, are also present in non-immobilised form.

7. A method as claimed in any one of claims 2 to 7, wherein the gene is the ryanodine receptor gene and the mutation is the halothane mutation.

8. A method as claimed in claim 8, wherein the terminal four nucleotides at the 3' ends of the immobilised primers are:

10 5'- GTCC -3' and  
5'- GGGT -3'.

9. A method of testing for the presence of the halothane mutation in a sample containing DNA from an animal, which method comprises,

15 performing an amplification reaction on the DNA using the PCR primers:

(i) 5'- MS-P1: 5'-CCT GTG TGT GTG CAA TGG TGT GGC  
CGT CC -3'

20 (ii) 5'- MS-P2: 5'-GTG CTG GAT GTC CTG TGT TCA ATG  
TGT GTG TGC AAT GGT GTG GCC GGG T -3'

and an antisense primer which hybridises to a DNA strand downstream of (i) and (ii), separating the amplification products and observing the separated products as an indication of the presence of one or more copies of the halothane mutation.

25 10. Apparatus for nucleic acid manipulations comprising a microtitre vessel, such as a microcentrifuge tube, having a closed lower end, to contain a volume of liquid which is small relative to the total internal volume of the vessel, and an open top which is closeable by a lid, the lid carrying a plug which extends into the tube and abuts the side wall thereof to define a liquid-containing region adjacent the lower end of the tube, wherein there is provided an insert mounted on the plug so as to be positioned in the said liquid-containing region of the

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tube, the insert being adapted to carry one or more immobilised nucleic acid deposits.

11. Apparatus as claimed in claim 10, wherein the plug reduces the internal volume of the vessel by at least half.

12. Apparatus as claimed in claim 10 or claim 11, which is suitable for performing the amplification reactions of any one of claims 1 to 10.

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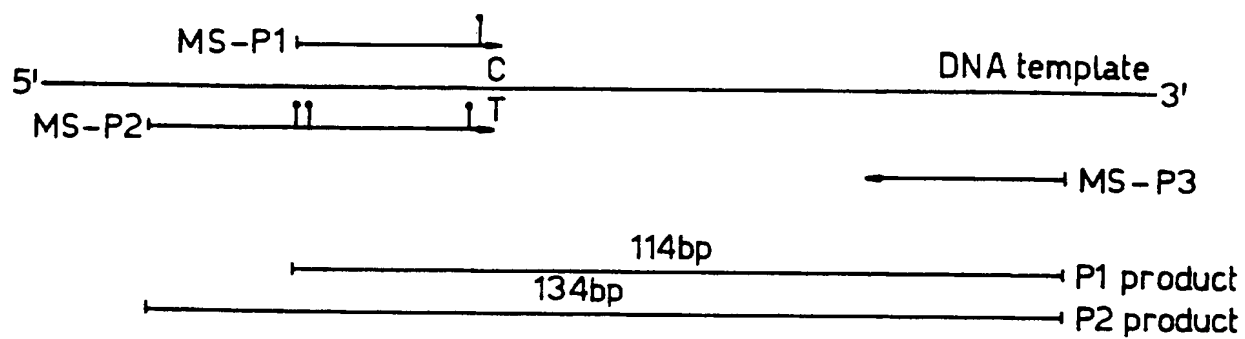
25

30

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Fig.1.



↑ introduced mutation

Fig.4.

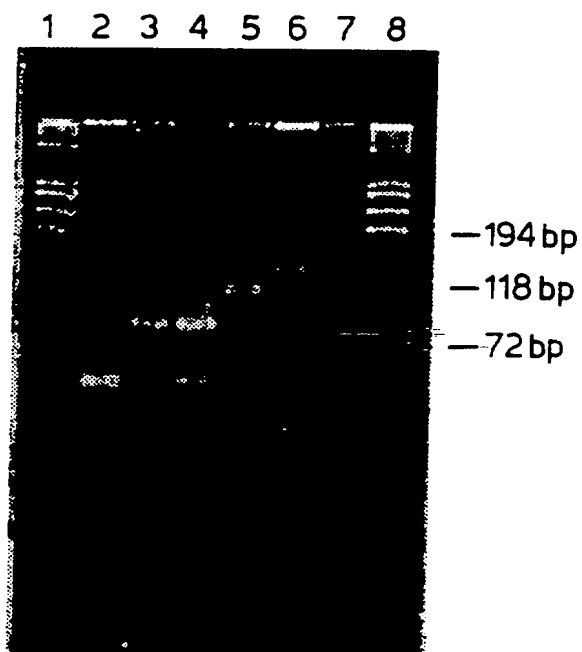


Fig.2.

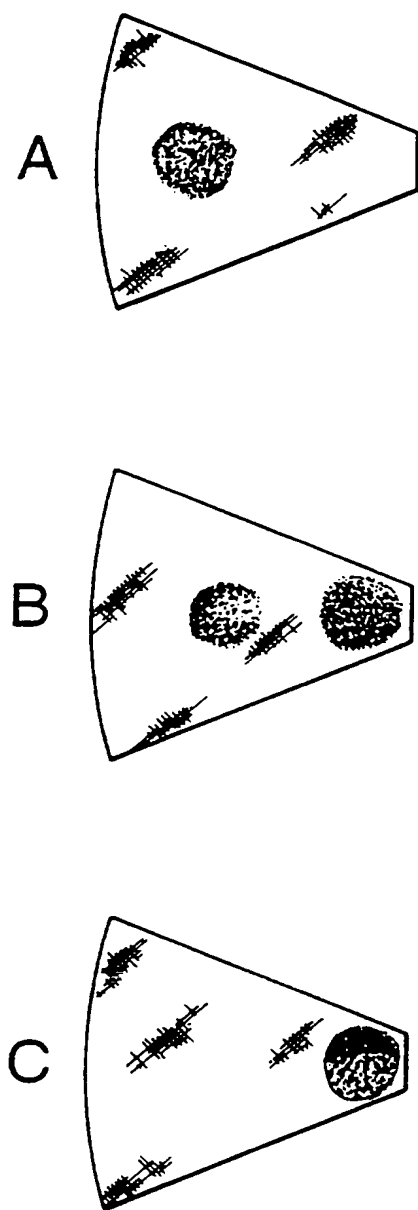
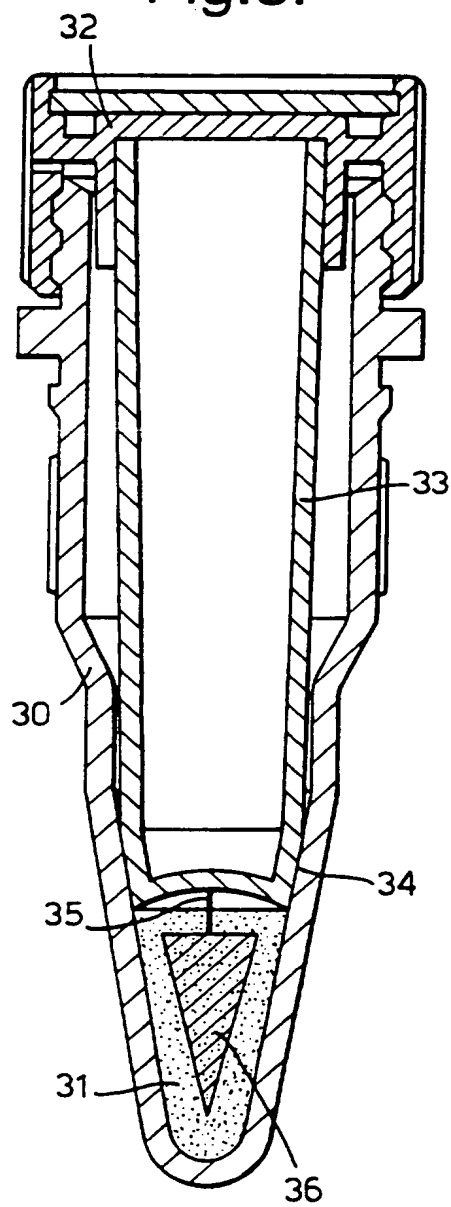


Fig.3.



## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 96/00420

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12Q1/68 B01L3/00 B01L3/14

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 530 794 (BOEHRINGER MANNHEIM GMBH) 10 March 1993 see the whole document ---	1-7
Y	EP,A,0 333 465 (BAYLOR COLLEGE OF MEDICINE) 20 September 1989 see the whole document ---	1-7
Y	WO,A,93 09250 (ADELAIDE CHILDREN'S HOSPITAL) 13 May 1993 see page 4, paragraph 2 - page 22; figure 4 ---	1-7
Y	WO,A,94 00597 (PHARMACIA LKB BIOTECHNOLOGY AB) 6 January 1994 see the whole document ---	1-7
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*&\* document member of the same patent family

Date of the actual completion of the international search

4 June 1996

Date of mailing of the international search report

07.08.96

Name and mailing address of the ISA

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Authorized officer

OSBORNE, H

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/GB 96/00420

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,94 09156 (THE REAGENTS OF THE UNIVERSITY OF CALIFORNIA) 28 April 1994 see page 10, paragraph 2 - page 13; examples VI-VII ---	1-7
A	WO,A,92 11387 (THE UNIVERSITY OF TORONTO INNOVATIONS FOUNDATION) 9 July 1992 see the whole document --- WO,A,96 04404 (MOSIAC TECHNOLOGIES) 15 February 1996 see the whole document --- WO,A,95 33073 (TEPNEL MEDICAL LTD) see the whole document -----	8,9

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB96/00420

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. Claims 1-9 : Method for detection of nucleic acid target sequences differing at a given locus
2. Claims 10-12: Apparatus for conducting solid phase nucleic acid manipulations/analysis

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-9

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 96/00420

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